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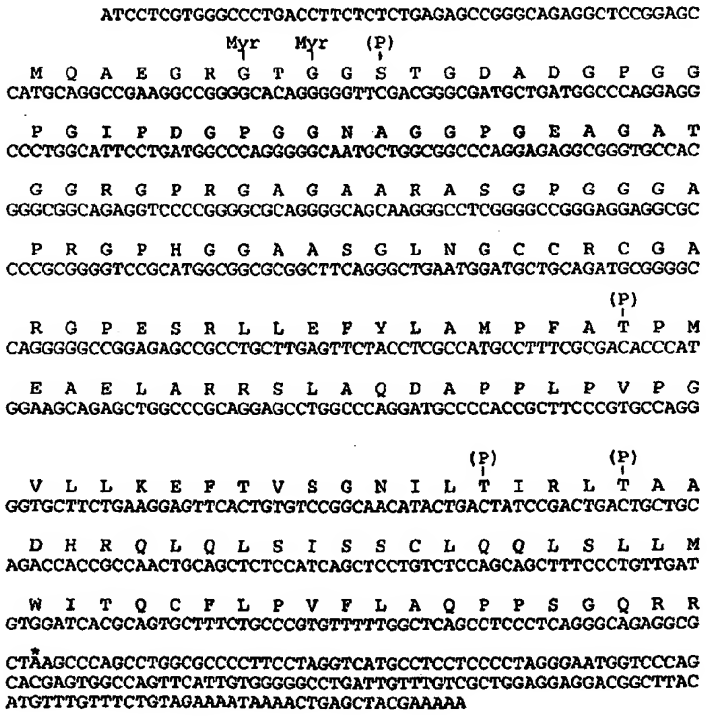
(54) Title: ISOLATED NUCLEIC ACID MOLECULE ENCODING CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF

(57) Abstract

The invention relates to the isolation of a nucleic acid molecule which encodes a cancer associated antigen (as shown in the figure). Also a part of the invention is the antigen itself, and the uses of the nucleic acid molecule and the antigen, and peptides derived from it.



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ISOLATED NUCLEIC ACID MOLECULE ENCODING CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF

RELATED APPLICATION

This is a continuation-in-part of Serial No. 08/725,182, filed October 3, 1996, and incorporated by reference.

FIELD OF THE INVENTION

This invention relates to an antigen associated with cancer, and the nucleic acid molecule encoding it, as well as the uses of these.

BACKGROUND AND PRIOR ART

It is fairly well established that many pathological conditions, such as infections, cancer, autoimmune disorders, etc., are characterized by the inappropriate expression of certain molecules. These molecules thus serve as "markers" for a particular pathological or abnormal condition. Apart from their use as diagnostic "targets", i.e., materials to be identified to diagnose these abnormal conditions, the molecules serve as reagents which can be used to generate diagnostic and/or therapeutic agents. A by no means limiting example of this is the use of cancer markers to produce antibodies specific to a particular marker. Yet another non-limiting example is the use of a peptide which complexes with an MHC molecule, to generate cytolytic T cells against abnormal cells.

Preparation of such materials, of course, presupposes a source of the reagents used to generate these. Purification from cells is one laborious, far from sure method of doing so. Another preferred method is the isolation of nucleic acid molecules which encode a particular marker, followed by the use of the isolated encoding molecule to express the desired molecule.

To date, two strategies have been employed for the detection of such antigens, in e.g., human tumors. These will be referred to as the genetic approach and the biochemical approach. The genetic approach is exemplified by, e.g., dePlaen et al., Proc. Natl. Sci. USA 85: 2275 (1988), incorporated by reference. In this approach, several hundred pools of plasmids of a cDNA library obtained from a tumor are transfected into recipient cells, such as COS cells, or into antigen-negative variants of tumor cell lines which are tested for the expression of the specific antigen. The biochemical approach, exemplified by, e.g., Kawakami, et al., Nature 369: 69 (1994) incorporated by reference, is based on acidic elution of peptides which have bound to MHC-class I molecules of tumor cells, followed by reversed-phase high performance liquid chromography (HPLC). Antigenic peptides are identified after they bind to empty MHC-class I molecules of mutant cell lines, defective in antigen processing, and induce specific reactions with cytotoxic T-lymphocytes. These reactions include induction of CTL proliferation, TNF release, and lysis of target cells, measurable in an MTT assay, or a ^{51}Cr release assay.

These two approaches to the molecular definition of antigens have the following disadvantages: first, they are enormously cumbersome, time-consuming and expensive; and second, they depend on the establishment of cytotoxic T cell lines (CTLs) with predefined specificity.

The problems inherent to the two known approaches for the identification and molecular definition of antigens is best demonstrated by the fact that both methods have, so far, succeeded in defining only very few new antigens in human tumors. See, e.g., van der Bruggen et al., Science 254: 1643-1647 (1991); Brichard et al., J. Exp. Med. 178: 489-495 (1993); Coulie, et al., J. Exp. Med. 180: 35-42 (1994); Kawakami, et al., Proc. Natl. Acad. Sci. USA 91: 3515-3519 (1994).

Further, the methodologies described rely on the availability of established, permanent cell lines of the cancer type under consideration. It is very difficult to establish cell lines from certain cancer types, as is shown by, e.g., Oettgen, et al., Immunol. Allerg. Clin. North. Am. 10: 607-637 (1990). It is also known that some epithelial cell type cancers are poorly susceptible to CTLs in vitro, precluding routine analysis. These problems have stimulated the art to develop additional methodologies for identifying cancer associated antigens.

One key methodology is described by Sahin, et al., Proc. Natl. Acad. Sci. USA 92: 11810-11913 (1995), incorporated by reference. Also, see U.S. Patent Applications Serial No. 08/580,980, and Application Serial No. 08/479,328, filed on June 7, 1995 and January 3, 1996, respectively. All three of these references are incorporated by reference. To summarize, the method involves the expression of cDNA libraries in a prokaryotic host. (The libraries are secured from a tumor sample). The expressed libraries are then immnoscreened with absorbed and diluted sera, in order to detect those antigens which elicit high titer humoral responses. This methodology is known as the SEREX method ("Serological identification of antigens by Recombinant Expression Cloning"). The methodology has been employed to confirm expression of previously identified tumor associated antigens, as well as to detect new ones. See the above referenced patent applications and Sahin, et al., supra, as well as Crew, et al., EMBO J 144: 2333-2340 (1995).

The SEREX methodology has been applied to esophageal cancer samples, and an antigen has now been identified, and its encoding nucleic acid molecule isolated and cloned. The antigen has been found to have more widespread distribution cancer, but had not been found in non-cancerous cells. This, inter alia, is the subject of the invention, which is described in more detail in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the expression pattern of RNA for the NY-ESO-1 antigen, in various tissue types.

Figure 2 shows Northern Blot analysis of NY-ESO-1 mRNA, which was found in testis and cell line SK-MEL-19, but not in various other cell and tissue samples.

Figure 3 shows potential sites for modification of the deduced amino acid sequence of NY-ESO-1.

Figure 4 is a hydrophilicity plot of NY-ESO-1, showing hydrophilic domains in the amino terminus and a long, hydrophobic stretch close to the carboxyl end.

Figure 5 shows the results of CTL lysis studies using various cells which are HLA-A2 positive, NY-ESO-1 positive, positive for both, or positive for neither.

Figure 6 presents data establishing that HLA-A2 is the presenting molecule for presentation of SEQ ID NO: 1 derived peptides.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

Total RNA was extracted from a snap frozen specimen of well to moderately differentiated squamous cell cancer of the esophagus, using well known methods. See, e.g., Chomzynski, J. Analyt. Biochem. 162: 156-159 (1987), for one such method. This RNA was used to prepare a cDNA library which was then transfected into λZAP phage vectors, in accordance with the manufacturer's instruction. The λZAP library was then transfected into E. coli, yielding 1.6×10^6 primary isolates.

The SEREX methodology of Sahin, et al., Proc. Natl. Acad. Sci. USA 92: 11810-11813 (1995), incorporated by reference, was then used. In brief, autologous serum was

stripped of antibodies against molecules which are endogenous to E. coli by combining the serum with lysates of E. coli transfected with phage λZAP which did not contain the cDNA clones from the esophageal cancer cells.

The depleted serum was then diluted, and mixed with nitrocellulose membranes containing phage plaques. The plaques were incubated overnight, at room temperature. Washing followed, and then the filters were incubated with alkaline phosphatase conjugated goat anti human FCγ secondary antibodies, and reactive phage plaques were visualized by incubating with 5-bromo-4-chloro-indolyl phosphate and nitroblue tetrazolium. A total of 13 positive clones were found.

Example 2

Following identification, the reactive clones were subcloned to monoclonality via dilution cloning and testing with human serum. These clones were then purified, excised in vitro, and converted into pBK-CMV plasmid forms, using the manufacturer's instructions. The inserted DNA was then evaluated using EcoRI-XbaI restriction mapping to determine different inserts. Eight different inserts were identified, ranging in size from about 500 to about 1.3 kilobase pairs. The clones were sequenced using an ABI PRISM automated sequencer.

Table 1 summarizes the results. One gene was represented by four overlapping clones, a second by three overlapping clones, and the remaining six by one clone only.

A homology search revealed that the clones referred to as NY-ESO-2, 3, 6, 7 were already known. See Elisei, et al., J. Endocrin. Invest. 16: 533-540 (1993); Spritz, et al., Nucl. Acids Res. 15: 10373-10391 (1987); Rabbits, et al., Nature Genetics 4: 175-180 (1993); Crozat, et al., Nature 363: 640-644 (1993); GenBank H18368 and D25606. Two of the clones (NY-ESO-3 and NY-ESO-6), have previously been shown to be expressed in various normal human tissues. No

evidence of lineage restriction has been found. NY-ESO-6 (cDNA), appears to be the 3'-untranslated portion of the FUS/TLS gene. In experiments not reported here, sequencing and Southern Blot analysis of NY-ESO-6 showed no evidence of translocation or point mutations in the cancer. Four of the clones, i.e., NY-ESO-1, 4, 5 and 8 showed no strong homology to sequences in the databases examined, and were thus studied further.

Table 1. Genes isolated from esophageal cancer library by immunoscreening with autologous serum

GENE	CLONE#	size	DNA databank	Comments*
NY-ESO-1	E1-5b E1-114b E1-153c E1-50	679bp 614bp 670bp 679bp	No strong homology	expressed in testis and ovary
NY-ESO-2	E1-71a E1-140 E1-31	605bp 874bp 750bp	U1 small nuclear RNP 1 homolog	cloned by Ab screening (thyroiditis patient)
NY-ESO-3	E1-141b	517bp	Colon 3' direct MboI cDNA; Adult brain cDNA	(dbj D25606, gb H18638) unpublished
NY-ESO-4	E1A-10c	400bp	No strong homology	ubiquitous expression in normal tissues
NY-ESO-5	E1A-54	670bp	No strong homology	expressed in normal esophagus
NY-ESO-6	E1B-9b	~1.2kb	Human *fus* mRNA	translocated in liposarcoma t(12;16)
NY-ESO-7	E1B-20f	~1.0kb	human U1-70k sn RNP	different from NY-ESO-2 (embl HSU17052, gbM22636)
NY-ESO-8	E1B-20g	~1.3kb	No strong homology	ubiquitous expression in normal tissues

Example 3

Studies were carried out to evaluate mRNA expression of the NY-ESO 1, 4, 5 and 8 clones. To do this, specific oligonucleotide primers were designed for each sequence, such that cDNA segments of 300-400 base pairs could be amplified, and so that the primer melting temperature would be in the range of 65-70°C. Reverse Transcription-PCR was then carried out using commercially available materials and standard protocols. A variety of normal and tumor cell types were tested. The clones NY-ESO-4 and NY-ESO-8 were ubiquitous, and were not studied further. NY-ESO-5 showed high level expression in the original tumor, and in normal esophageal tissue, suggesting that it was a differentiation marker.

NY-ESO-1 was found to be expressed in tumor mRNA and in testis, but not normal colon, kidney, liver or brain tissue. This pattern of expression is consistent with other tumor rejection antigen precursors.

Example 4

The RT-PCR assay set forth supra was carried out for NY-ESO-1 over a much more complete set of normal and tumor tissues. Tables 2, 3 and 4 show these results. In brief, NY-ESO-1 was found to be highly expressed in normal testis and ovary cells. Small amounts of RT-PCR production were found in normal uterine myometrium, and not endometrium, but the positive showing was not consistent. Squamous epithelium of various cell types, including normal esophagus and skin, were also negative.

When tumors of unrelated cell lineage were tested, 2 of 11 melanomas cell lines showed strong expression, as did 16 of 67 melanoma specimens, 6 of 33 breast cancer specimens and 4 of 4 bladder cancer. There was sporadic expression in other tumor types.

Table 2: mRNA distribution of NY-ESO-1 in normal tissues

Tissue	mRNA	Tissue	mRNA
Esophagus	-	Adrenal	-
Brain*	-	Pancreas	-
Fetal Brain	-	Seminal Vesicle	-
Heart	-	Placenta	-
Lung	-	Thymus	-
Liver	-	Lymph node	-
Spleen	-	Tonsil	-
Kidney	-	PBL	-
Stomach	-	PBL, activated#	-
Small intestine	-	Melanocytes	-
Colon	-	Thyroid	-
Rectum	-	Uterus	+/-**
Breast	-	Testis	+
Skin	-	Ovary	+

*tissues from several parts tested #with IL-2 and PHA
**weakly positive in some specimens, negative by Northern blot

Table 3: mRNA distribution of NY-ESO-1 in melanoma and breast cancer cell lines:

Cell line	NY-ESO-1 mRNA
MZ2-MEL3.1	-
MZ2-MEL2.2	-
SK-MEL-13	-
SK-MEL-19	+
SK-MEL-23	-
SK-MEL-29	-
SK-MEL-30	-
SK-MEL-31	-
SK-MEL-33	-
SK-MEL-37	+
SK-MEL-179	-
SK-BR-3	-
SK-BR-5	-
734B	-
MDA-MB-231	-

Table 4: NY-ESO-1 mRNA expression in various human tumors by RT-PCR

tumor type	mRNA(positive/total)	tumor type	mRNA(positive/total)
melanoma	25/77	ovarian cancer	2/8
breast cancer	17/43	thyroid cancer	2/5
prostate cancer	4/16	bladder cancer	9/13
colon cancer	0/16	Burkitt's lymphoma	1/2
glioma	0/15	basal cell carcinoma	0/2
gastric cancer	0/12	Jejomyosarcoma	0/2
lung cancer	5/17	other sarcomas	0/2
renal cancer	0/10	pancreatic cancer	0/2
lymphoma*	0/10	seminoma	0/1
hepatoma	2/7	spinal cord tumor	0/1

*non-Hodgkin's, non-Burkitt's types.

A further set of experiments were carried out to ascertain if the presence of anti NY-ESO-1 antibody in cancer patient sera could be determined via an ELISA. In brief, recombinant NY-ESO-1 protein, produced via the methods set forth infra, was coated onto solid surfaces, using standard methods. Serum samples were taken from normal patients and patients with various cancers. Anti NY-ESO-1 antibodies should bind thereto. These were then contacted with goat-anti human IgG, labelled with alkaline peroxidase, and then visualized with substrate. The results are presented in the following table:

	Eso 1 +/total tested	%
Cancer patients:		
melanoma	11/127	8.7
ovarian cancer	4/31	12.9
lung cancer	1/24	4.0
Blood donors:	0/70	0

Example 5

Northern blot analysis was then carried out to investigate
the size of the NY-ESO-1 transcript, and to confirm tissue expression patterns. The methodology of Ausubel, et al., Current Protocols In Molecular Biology (John Wiley & Sons, 1995) was used. To be specific, 20 ug of total RNA per lane were dissolved in a formamide and formaldehyde containing buffer, heated to 65°C, and then separated on a 1.2% agarose gel, with 3% formaldehyde, followed by transfer to nitrocellulose paper. Hybridization was then carried out using a ^{32}P labelled probe, followed by high stringency washing. The final wash was at 0.1xSSC, 0.1% SDS, 60°C, for 15 minutes.

RNA from testis, and a melanoma cell line (SK-MEL-19) which had been positive for NY-ESO-1 in the prior assays, showed an RNA transcript of about 0.8-0.9 kb. An esophageal carcinoma specimen showed a smear in the 0.4-0.9

kb range, reflecting partial degradation. RNA from additional tissues or cell lines tested showed no transcript.

To get cDNA encoding the full transcript, the esophageal cDNA library was rescreened, using plaque hybridization, and the original cDNA clone as the hybridization probe. When $3x10^5$ clones were screened, six positives were found. The three longest clones were sequenced. Analysis of open reading frames showed that all three contained the entire coding region, and 5'-untranslated regions of variable size. The longest clone, 755 base pairs in length, (excluding polyA), contains a 543 base pair coding region, together with 53 untranslated bases at the 5' end and 151 untranslated base pairs at the 3'-end. See SEQ ID NO: 1 (also, Figure 3).

The long ORF indicated that the deduced sequence of NY-ESO-1 protein is 180 amino acids. The single immunopositive clone contained a sequence encoding 173 of these. Deduced molecular mass is 17,995 daltons.

Analysis shows that there is an abundance of glycine residues in the N-terminal portion (30 of the first 80, 4 in the remaining 100). Hydrophilicity analysis indicated that there were hydrophilic antigenic sequences in the N-terminal half of the molecule, with alternating hydrophobic and hydrophilic sequences, ending with a long, C-terminal hydrophobic tail (amino acids 152-172), followed by a short hydrophilic tail. This pattern suggests a transmembrane domain. There are several potential N-myristorylation sites, 3 phosphorylation sites, and no evidence of N-glycosylation sites.

Example 6

A melanoma cell line "NW-MEL-38" was established, in 1995, from a patient who suffered from malignant melanoma. Serum samples, peripheral blood lymphocytes, and tumor samples, were taken from the subject and frozen, until the work described herein was carried out. In anticipation of

evaluating antitumor T cell response in this patient, the patient was HLA typed as HLA-A1 and HLA-A2.

To determine whether melanoma from this patient expressed NY-ESO-1, total RNA was isolated from both tumor samples and cell line NW-MEL-38, using standard techniques. Then, two micrograms of the total RNA, from each samples were subjected to cDNA synthesis, again using standard techniques.

The cDNA was then used in RT-PCR experiments, using the following primers:

5'-CACACAGGAT CCATGGATGC TGCAGATGCG G'-3' (SEQ ID NO: 2), and

CACACAAAGC TTGGCTTAGC GCCTCTGCCC TG-3' (SEQ ID NO: 3)

These primers should amplify a segment of SEQ ID NO: 1 which spans nucleotides 271 to 599.

Amplification was carried out over 35 cycles, using an annealing temperature of 60°C. The PCR products were visualized via ethidium bromide staining, on a 1.5% agarose gel.

The results indicated that both the tumor and the cell line expressed SEQ ID NO: 1. The cell line and tumor samples were used in subsequent experiments.

Example 7

The isolated cDNA molecule, discussed supra, was then used to make recombinant protein. Specifically, the cDNA was PCR amplified, using standard techniques, and was then cloned into a commercially available plasmid vector, i.e., pQE9, which contains His tags. In work not elaborated upon herein, a second vector, pQE9K was also used. This differs from PQE9 in that kanamycin resistance is imparted by pQE9K, rather than ampicillin resistance.

The plasmid vector was transformed into E. coli strain XL1-Blue, and positive transformants were identified via restriction mapping and DNA sequencing. Production of

recombinant protein was induced using isopropyl ß-D-thiogalactoside, and the protein was purified on an Ni^{2+} ion chromatography column, following well known procedures. The protein when analyzed via 15% SDS-PAGE and silver staining, was identified as a protein with a molecular weight of about 22 kilodaltons. This is consistent with the anticipated size of the protein from its sequence.

Eukaryotic transfectants were then produced. To do this, the NY-ESO-1 coding sequence was isolated from the pQE9 vector described supra, and then cloned into BamHI-HindIII sites of eukaryotic expression vector pcDNA 3.1. Next, COS-7 cells were transfected with this vector, by contacting cell samples with 150 ng of the plasmid discussed supra, and 150 ng of plasmid pcDNA 1 Amp, which contained either cDNA for HLA-A2.1 or cDNA for HLA-A1, The well known DEAE-dextran chloroquine method was used. The cells were then incubated at 37°C, for 48 hours, after which they were tested in a CTL stimulation assay. Specifically, the assay followed Traversari et al, Immunogenetics 35: 145-148 (1992), incorporated by reference. In brief, 2500 CTLs, (NW38-IVS-1, see example 9, infra), in 100 ul RPMI supplemented with 100% human serum, and 25 U/ml of recombinant IL-2 were added to microwells containing COS-7 transfectants (20,000 cells/well). After 24 hours, 50 ul of supernatant were collected from each well, and TNF-α levels were determined in a standard assay, i.e., one where cytotoxicity against WEHI 164 clone 13 cells were tested, using MTT. Positive cells were used in the Western Blot analysis, described in the example which follows.

The CTLs used were CTL NW38-IVS-1, prepared in accordance with Knuth et al., Proc. Natl. Acad. Sci. USA 81: 3511-3515 (1984), incorporated by reference. Specifically, mixed lymphocyte T cell cultures were set up, by combining 10^5 autologous NW38 MEL-1 tumor cells, and 10^6 peripheral blood lymphocytes, taken from the subject. The cytokine IL-2 was added, and the mixed culture was

incubated for one week at 37°C. Tumor cells were removed, and a new aliquot of $5x10^4$ tumor cells were added together with IL-2. This process was repeated weekly, until a strong response was seen when tested against ^{51}Cr labelled NW-MEL-38 cells. The responder T cells were collected and frozen until used in further experiments.

Example 8

Western Blot analysis was then carried out, using the serum samples described supra, as well as cell lysates taken from the cell line NW-MEL-38, described supra, and the COS-7 transfectants, described supra, and the purified recombinant protein, also described supra. Serum samples were taken from various points of the patient's therapy. There was no difference in the results.

In these assays, 1 ug of recombinant NY-ESO-1 protein, or 5 ul of cell lysates of either type were diluted in SDS and boiled for five minutes, and then electrophoresed on a 15% SDS gel. After overnight blotting on nitrocellulose (0.45 um), and blocking with 3% BSA, the blots were incubated with serum, diluted at 1:1000, 1:10,000, and 1:100,000, or with a monoclonal antibody against NY-ESO-1, diluted to 1:50, as a positive control. The monoclonal antibody was prepared as follows. BALB/C mice were immunized via five subcutaneous injections of recombinant NY-ESO-1 protein, at 2-3 week intervals. The immunizing formulation included 50 ug of recombinant protein in adjuvant. The first injection used Complete Freund's Adjuvant, and Incomplete Freund's Adjuvant was used thereafter. Spleen cells were taken from the immunized mice, and fused with mouse myeloma cell line SP2/0, to generate hybridomas.

Once hybridomas were generated, they were cloned, and their supernants were screened against recombinant protein, using a standard solid phase ELISA on microtiter plates. The assay was in accordance with Dippold et al., Proc. Natl. Acad. Sci. USA 77: 6114-6118 (1980), incorporated by

reference. A series of negative controls were also run, using recombinant NY-ESO-1. Serum antibodies which bound to recombinant protein, produced by E. coli as described, supra were visualized using goat anti-human IgG, labelled with alkaline phosphatase at 1:10,000 dilution, and were then visualized with NBT-phosphate. Untransfected COS-7 cells were also used as a control. Serum from a healthy individual was also used as a control.

Strong reactivity against the recombinant protein was found at serum dilutions down to 1:100,000, and there was also reactivity against lysate of NW-MEL-38. There was no reactivity found against the untransfected COS-7 cells, nor did the serum from a healthy individual show reactivity.

Example 9

In the testing of the COS-7 transfectants, supra, and the assays discussed in this example, a cytolytic T cell line "NW38-IVS-1" was used. This "CTL" was generated, via in vitro stimulation of the peripheral blood lymphocytes mentioned supra, using the tumor cell line NW-MEL-38. This was done using standard techniques.

The CTL was used in a cytotoxicity assay with NW-MEL-38 (which was HLA-A1, A2 positive, and NY-ESO-1 positive), along with two allogeneic cell lines which were NY-ESO-1 and HLA-A2 positive (SK-MEL-37 and MZ-MEL-19), a cell line which is MHC Class I negative (SK-MEL-19), a cell line which is HLA-A2 positive, but NY-ESO-1 negative (NW-MEL-145), along with control cell lines K562 and autologous phytohemagglutinin stimulated blasts. Various effector/target ratios were used, and lysis of ^{51}Cr labelled target cells was the parameter measured. Figure 5 shows this.

The results indicated that the CTL NW38-IVS-1 lysed both the autologous cell line NW MEL-38, and the allogeneic cell lines which were HLA-A2 and ESO-1 positive. Hence, the CTL was reactive with allogeneic materials. See figure 6.

Example 10

As patient NW38 was HLA-A1 and HLA-A2 positive experiments were carried out to determine which MHC molecule was the presenting molecule.

The same experiment, described supra with COS-7 cells was carried out, except that, in these experiments, care was taken to secure separate groups of cotransformants which had been transformed with either HLA-A1 cDNA, or HLA-A2 cDNA, but not both. These results show that the CTL NW38-IVS-1 lysed COS-7 transfectants containing both NY-ESO-1 and HLA-A2 exclusively. See figure 6. The work also confirmed the specificity of the CTL, since the NY-ESO-1 negative, HLA-A2 positive cells described in Example 9 were positive for other molecules known to be processed to peptides presented by HLA-A2 molecules.

Example 11

Once the presenting MHC Molecule was identified as HLA-A2, a screening of the amino acid sequence for NY-ESO-1 was carried out, to identify all peptides which satisfy this motif, using the model set forth by D'Amaro et al., Human Immunol. 43: 13-18 (1995), and Drijfhout, et al., Human Immunol. 43: 1-12 (1995) incorporated by reference. Peptides corresponding to all of the amino acid sequences deduced thereby were synthesized, using standard techniques, and were then used in cytotoxicity assays, following Knuth et al., Proc. Natl. Acad. Sci. USA 81: 3511-3515 (1984), incorporated by reference. Specifically, cell line CEMX721.174.T2 ("T2" hereafter), was used, because it does not process antigens to MHC complexed peptides, thereby making it ideal for experiments of the type described herein. Samples of T2 cells were labelled with 100 uCi of $Na(^{51}Cr)O_4$, using standard methods, and were then washed three times, followed by incubation with 10 ug/ml peptide and 2.5 ug/ml of ß2-microglobulin. Incubation was for one hour, at room temperature. Then

responder cells (100 ul of a suspension of CTL NW38-IVS-1) were added, at an effector/target ratio of 90:1, and incubated for four hours in a water saturated atmosphere, with 5% CO_2, at 37°C. Then, plates were centrifuged at 200xg for five minutes, 100 ul of supernatant was removed, and radioactivity was measured. The percentage of ^{51}Cr release was determined in accordance with known strategies. it was found that the peptides SLLMWITQCFL (SEQ ID NO: 4), SLLMWITQC (SEQ ID NO: 5), and QLSLLMWIT (SEQ ID NO: 6), were the three best stimulators of CTLs. Comparable results were found when NW-MEL-38 and cell lines SK-MEL-37 and MZ-MEL-19 were used as targets, as is shown, supra.

The foregoing examples describe the isolation of a nucleic acid molecule which encodes an esophageal cancer associated antigen. "Associated" is used herein because while it is clear that the relevant molecule was expressed by esophageal cancer, other cancers, such as melanoma, breast, prostate and lung also express the antigen.

The invention relates to those nucleic acid molecules which encode antigens as described, and which hybridize to reference sequence SEQ ID NO: 1 under stringent conditions. "Stringent conditions" as used herein refers to conditions such as those specified in U.S. Patent No. 5,342,774, i.e., 18 hours of hybridization at 65°C, followed by four one hour washes at 2xSSC, 0.1% SDS, and a final wash at 0.2xSSC, more preferably 0.1xSSC, 0.1% SDS for 30 minutes, as well as alternate conditions which afford the same level of stringency, and more stringent conditions.

Also a part of the invention are expression vectors which incorporate the nucleic acid molecules of the invention, in operable linkage (i.e., "operably linked") to a promoter. Construction of such vectors is well within the skill of the art, as is the transformation or transfection of cells, to produce eukaryotic cell lines, or prokaryotic cell strains which encode the molecule of interest. Exemplary of the host cells which can be employed in this fashion are COS cells, CHO cells, yeast

cells, insect cells (e.g., Spodoptera frugiperda), NIH 3T3 cells, and so forth. Prokaryotic cells, such as E. coli and other bacteria may also be used.

Also a part of the invention is the antigen described herein, both in original peptide form and in post translational modified form. The molecule is large enough to be antigenic without any posttranslational modification, and hence it is useful as an immunogen, when combined with an adjuvant (or without it), in both precursor and post-translationally modified forms. The antibodies produced using this antigen, both poly and monoclonal, are also a part of the invention as well as hybridizing which make the monoclonal antibody. It can be used therapeutically as the whole protein, or in portions, as discussed infra. Also a part of the invention are antibodies against this antigen, be these polyclonal, monoclonal, reactive fragments, such as Fab, $F(ab)_2'$ and other fragments, as well as chimeras, humanized antibodies, recombinantly produced antibodies, and so forth.

As is clear from the disclosure, one may use the proteins and nucleic acid molecules of the invention diagnostically. The SEREX methodology discussed herein is premised on an immune response to a pathology associated antigen. Hence, one may assay for the relevant pathology via, e.g., testing a body fluid sample of a subject, such as serum, for reactivity with the antigen per se. Reactivity would be deemed indicative of possible presence of the pathology so, too, could one assay for the expression of the antigen via any of the standard nucleic acid hybridization assays which are well known to the art, and need not be elaborated upon herein. One could assay for antibodies against the subject molecule, using standard immunoassays as well.

Analysis of SEQ ID NO: 1 will show that there are 5' and 3' non coding regions presented therein. The invention relates to those isolated nucleic acid molecules which contain at least the coding segment, i.e., nucleotides 54-

593, and which may contain any or all of nucleotides 1-53 and/or 594-747 of SEQ ID NO: 1.

Further analysis, as discussed supra, reveals that the molecule is processed to peptides which provoke lysis by cytolytic T cells. Example 7 showed how this type of motif analysis can be carried out for HLA-A2 molecules. There has been a great deal of work in motifs for various MHC or HLA molecules, which is applicable here. Hence, a further aspect of the invention is a therapeutic method, wherein one or more peptides which bind to an HLA molecule on the surface of a patient's tumor cells are administered to the patient, in an amount sufficient for the peptides to bind to the MHC/HLA molecules, and provoke lysis by T cells. The exemplification given supra for HLA-A2 molecules is by no means the only type of this administration that can be used. Any combination of peptides may be used. These peptides, which may be used alone or in combination, as well as the entire protein or immunoreactive portions thereof, may be administered to a subject in need thereof, using any of the standard types of administration, such as intravenous, intradermal, subcutaneous, oral, rectal, and transdermal administration. Standard pharmaceutical carriers, adjuvants, such as saponins, GM-CSF, and interleukins and so forth may also be used. Further, these peptides and proteins may be formulated into vaccines with the listed material, as may dendritic cells, or other cells which present relevant MHC/peptide complexes.

Similarly, the invention contemplates therapies wherein the nucleic acid molecule which encodes NY-ESO-1 is incorporated into a vector, such as an adenovirus based vector, to render it transfectable into eukaryotic cells, such as human cells. Similarly, nucleic acid molecules which encode one or more of the peptides may be incorporated into these vectors, which are then the major constituent of nucleic acid bases therapies.

Any of these assays can also be used in progression/regression studies. One can monitor the course

of abnormality involving expression of NY-ESO-1, simply by monitoring levels of the protein, its expression, and so forth using any or all of the methods set forth supra.

It should be clear that these methodologies may also be used to track the efficacy of a therapeutic regime. Essentially, one can take a baseline value for the NY-ESO-1 protein, using any of the assays discussed supra, administer a given therapeutic agent, and then monitor levels of the protein thereafter, observing changes in ESO-1 levels as indicia of the efficacy of the regime.

As was indicated supra, the invention involves, inter alia, the recognition of an "integrated" immune response to the NY-ESO molecule. One ramification of this is the ability to monitor the course of cancer therapy. In this method, which is a part of the invention, a subject in need of the therapy receives a vaccination of a type described herein. Such a vaccination results, e.g., in a T cell response against cells presenting HLA/peptide complexes on their cells. The response also includes an antibody response, possibly a result of the release of antibody provoking proteins via the lysis of cells by the T cells. Hence, one can monitor the effect of a vaccine, by monitoring an antibody response. As is indicated, supra, an increase in antibody titer may be taken as an indicia of progress with a vaccine, and vice versa. Hence, a further aspect of the invention is a method for monitoring efficacy of a vaccine, following administration thereof, by determining levels of antibodies in the subject which are specific for the vaccine itself, or a large molecules of which the vaccine is a part.

The identification of NY-ESO-1 proteins as being implicated in pathological conditions such as cancer also suggests a number of therapeutic approaches in addition to those discussed supra. The experiments set forth supra establish that antibodies are produced in response to expression of the protein. Hence, a further embodiment of the invention is the treatment of conditions which are

characterized by aberrant or abnormal levels of NY-ESO-1 proteins, via administration of antibodies, such as humanized antibodies, antibody fragments, and so forth. These may be tagged or labelled with appropriate cystostatic or cytotoxic reagents.

T cells may also be administered. It is to be noted that the T cells may be elicited in vitro using immune responsive cells such as dendritic cells, lymphocytes, or any other immune responsive cells, and then reperfused into the subject being treated.

Note that the generation of T cells and/or antibodies can also be accomplished by administering cells, preferably treated to be rendered non-proliferative, which present relevant T cell or B cell epitopes for response, such as the epitopes discussed supra.

The therapeutic approaches may also include antisense therapies, wherein an antisense molecule, preferably from 10 to 100 nucleotides in length, is administered to the subject either "neat" or in a carrier, such as a liposome, to facilitate incorporation into a cell, followed by inhibition of expression of the protein. Such antisense sequences may also be incorporated into appropriate vaccines, such as in viral vectors (e.g., Vaccinia), bacterial constructs, such as variants of the known BCG vaccine, and so forth.

Also a part of the inventions are peptides, which can be nonamers, decamers, or undecamers defined as having a core sequence:

LLMWIT (SEQ ID NO: 7)

which have at least one additional residue terminal to the first L residue, preferably serine and may have as many as three, wherein Serine is linked to L to form -SL-, and 0-4 additional amino acids at the C-terminus which, as shown supra, bind to HLA-A2 molecules, thereby provoking a CTL response. These peptides may be used therapeutically, via administration to a patient who is HLA-A2 positive, and expresses NY-ESO-1 in connection with a pathology, as well

as diagnostically, i.e., to determine if HLA-A2 positive cells are present, or if relevant CTLs are present, and so forth.

The HLA-A2 molecule is an MHC Class I molecule, and T cells which respond to complexes of peptides and class I molecules are generally $CD8^+$ cells. Another subset of T cells, $CD4^+$ cells, responds to complexes of MHC-Class II molecules and peptides, and MHC-Class II restricted $CD4^+$ T cell responses against recombinant NY-ESO-1, presented by autologous cultured dendritic cells have been detected in melanoma patients. Specifically, in results not described herein, $CD4^+$ cells were separated from other cells from PBLs or serum samples, using well known techniques. Then, they were admixed with dendritic cells which had been pulsed with NY-ESO-1 protein. Proliferation of $CD4^+$ cells was observed, bringing another facet to the integrated immune response discussed herein. Hence, a further aspect of this invention are these $CD4^+$ T cells, peptides which bind to the MHC-Class II molecules, and their use in therapy.

Other features and applications of the invention will be clear to the skilled artisan, and need not be set forth herein.

The terms and expression which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expression of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: ISOLATED NUCLEIC ACID MOLECULE ENCODING AN ESOPHAGEAL CANCER ASSOCIATED ANTIGEN, THE ANTIGEN ITSELF, AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 7

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(A) MEDIUM TYPE: Diskette, 3.5 inch, 144 kb storage

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 752 base pairs
(B) TYPE: nuclear acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATCCTCGTGG GCCCTGACCT TCTCTCTGAG AGCCGGGCAG AGGCTCCGGA GCC          53

ATG CAG GCC GAA GGC CGG GGC ACA GGG GGT TCG ACG GGC GAT GCT          98
Met Gln Ala Glu Gly Arg Gly Thr Gly Gly Ser Thr Gly Asp Ala
                  5                  10                  15

GAT GGC CCA GGA GGC CCT GGC ATT CCT GAT GGC CCA GGG GGC AAT         143
Asp Gly Pro Gly Gly Pro Gly Ile Pro Asp Gly Pro Gly Gly Asn
                 20                  25                  30

GCT GGC GGC CCA GGA GAG GCG GGT GCC ACG GGC GGC AGA GGT CCC         188
Ala Gly Gly Pro Gly Glu Ala Gly Ala Thr Gly Gly Arg Aly Pro
                 35                  40                  45

CGG GGC GCA GGG GCA GCA AGG GCC TCG GGG CCG GGA GGA GGC GCC         233
Arg Gly Ala Gly Ala Ala Arg Ala Ser Gly Pro Gly Gly Gly Ala
                 50                  55                  60

CCG CGG GGT CCG CAT GGC GGC GCG GCT TCA GGG CTG AAT GGA TGC         278
Pro Arg Gly Pro His Gly Gly Ala Ala Ser Gly Leu Asn Gly Cys
                 65                  70                  75

TGC AGA TGC GGG GCC AGG GGG CCG GAG AGC CGC CTG CTT GAG TTC         323
Cys Arg Cys Gly Ala Arg Gly Pro Glu Ser Arg Leu Leu Glu Phe
                 80                  80                  90

TAC CTC GCC ATG CCT TTC GCG ACA CCC ATG GAA GCA GAG CTG GCC         368
Tyr Leu Ala Met Pro Phe Ala Thr Pro Met Glu Ala Glu Leu Ala
                 95                 100                 105

CGC AGG AGC CTG GCC CAG GAT GCC CCA CCG CTT CCC GTG CCA GGG         413
Arg Arg Ser Leu Ala Gln Asp Ala Pro Pro Leu Pro Val Pro Gly
                110                 115                 120

GTG CTT CTG AAG GAG TTC ACT GTG TCC GGC AAC ATA CTG ACT ATC         458
Val Leu Leu Lys Glu Phe Thr Val Ser Gly Asn Ile Leu Thr Ile
                125                 130                 135

CGA CTG ACT GCT GCA GAC CAC CGC CAA CTG CAG CTC TCC ATC AGC         503
Arg Leu Thr Ala Ala Asp His Arg Gln Leu Gln Leu Ser Ile Ser
                140                 145                 150

TCC TGT CTC CAG CAG CTT TCC CTG TTG ATG TGG ATC ACG CAG TGC         548
Ser Cys Leu Gln Gln Leu Ser Leu Leu Met Trp Ile Thr Gln Cys
                155                 160                 165

TTT CTG CCC GTG TTT TTG GCT CAG CCT CCC TCA GGG CAG AGG CGC         593
Phe Leu Pro Val Phe Leu Ala Gln Pro Pro Ser Gly Gln Arg Arg
                170                 175                 180

TAA GCCCAGCCTG GCGCCCCTTC CTAGGTCATG CCTCCTCCCC TAGGGAATGG         646

TCCCAGCACG AGTGGCCAGT TCATTGTGGG GGCCTGATTG TTTGTCGCTG GAGGAGGACG  706

GCTTACATGT TTGTTTCTGT AGAAAATAAA ACTGAGCTAC GAAAAA                 752
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nuclear acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CACACAGGAT CCATGGATGC TGCAGATGCG G 31

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs
(B) TYPE: nuclear acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CACACAAAGC TTGGCTTAGC GCCTCTGCCC TG 32

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ser Leu Leu Met Trp Ile Thr Gln Cys Phe Leu
5 10

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ser Leu Leu Met Trp Ile Thr Gln Cys
5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gln Leu Ser Leu Leu Met Trp Ile Thr
5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Leu Leu Met Trp Ile Thr
5

We claim:

1. Isolated nucleic acid molecule which encodes a cancer associated antigen, the complementary sequence of which hybridizes, under stringent conditions, to the nucleic acid molecule having the nucleotide sequence set forth in nucleotides 54-593 of SEQ ID NO: 1.

2. The isolated nucleic acid molecule of claim 1, consisting of nucleotides 54-593 of SEQ ID NO: 1.

3. The isolated nucleic acid molecule of claim 1, consisting of anywhere from nucleotide 1 through nucleotide 747 of SEQ ID NO: 1, with the proviso that said isolated nucleic acid molecule contains at least nucleotides 54-593 of SEQ ID NO: 1.

4. Expression vector comprising the isolated nucleic acid molecule of claim 1, operably linked to a promoter.

5. Expression vector comprising the isolated nucleic acid molecule of claim 3, operably linked to a promoter.

6. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 4.

7. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 5.

8. Isolated cancer associated antigen comprising all or part of the amino acid sequence encoded by nucleotides 54-593 of SEQ ID NO: 1.

9. Eukaryotic cell line or prokaryote cell strain, transformed or transfected with the isolated nucleic acid molecule of claim 1.

10. The eukaryotic cell line of claim 9, wherein said cell line is also transfected with a nucleic acid molecule coding for a cytokine.

11. The eukaryotic cell line of claim 10, wherein said cell line is further transfected by a nucleic acid molecule coding for an HLA molecule.

12. The eukaryotic cell line of claim 10, wherein said cytokine is an interleukin.

13. The biologically pure culture of claim 12, wherein said interleukin is IL-2, IL-4 or IL-12.

14. The eukaryotic cell line of claim 9, wherein said cell line has been rendered non-proliferative.

15. The eukaryotic cell line of claim 9, wherein said cell line is a fibroblast cell line.

16. Expression vector comprising a mutated or attenuated virus and the isolated nucleic acid molecule of claim 1.

17. The expression vector of claim 16, wherein said virus is adenovirus.

18. The expression vector of claim 4, further comprising a nucleic acid molecule which codes for an MHC or HLA.

19. The expression vector of claim 4, further comprising a nucleic acid molecule which codes for a cytokine.

20. The expression vector of claim 4, wherein said cytokine is interleukin.

21. The expression vector of claim 20, wherein said interleukin is IL-2, IL-4 or IL-12.

22. The expression vector of claim 16, wherein said virus is vaccinia virus.

23. Expression system useful in transfecting a cell, comprising (i) a first vector containing a nucleic acid molecule which codes for the isolated cancer associated antigen of claim 8 and (ii) a second vector selected from the group consisting of (a) a vector containing a nucleic acid molecule which codes for an MHC or HLA molecule which presents an antigen derived from said cancer associated antigen and (b) a vector containing a nucleic acid molecule which codes for an interleukin.

24. Isolated cancer associated antigen comprising the amino acid sequence encoded by nucleotides 54-593 of SEQ ID NO: 1.

25. Immunogenic composition comprising the isolated antigen of claim 24, and a pharmaceutically acceptable adjuvant.

26. The immunogenic composition of claim 25, wherein said adjuvant is a cytokine, a saponin, or GM-CSF.

27. Immunogenic composition comprising at least one peptide consisting of an amino acid sequence of from 8 to 12 amino acids concatenated to each other in the isolated cancer associated antigen of claim 24, and a pharmaceutically acceptable adjuvant.

28. The immunogenic composition of claim 27, wherein said adjuvant is a saponin, a cytokine, or GM-CSF.

29. The immunogenic composition of claim 27, wherein said composition comprises a plurality of peptides which complex with a specific MHC molecule.

30. The immunogenic composition of claim 29, wherein said MHC molecule is HLA-A2.

31. Isolated peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, wherein said isolated peptide binds to an HLA molecule, is a nonamer, decamer or undecamer, and comprises the amino acid sequence of SEQ ID NO: 7, from one to three additional N-terminal amino acid, and up to four additional C terminal amino acids.

32. The isolated peptide of claim 31, wherein said HLA molecule is HLA-A2.

33. The isolated peptide of claim 31, selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 6.

34. Immunogenic composition which comprises at least one expression vector which encodes for a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, and an adjuvant or carrier.

35. The immunogenic composition of claim 34, wherein said at least one expression vector codes for a plurality of peptides.

36. Vaccine useful in treating a subject afflicted with a cancerous condition comprising the isolated cell line of claim 11 and a pharmacologically acceptable adjuvant.

37. The vaccine of claim 36, wherein said cell line has been rendered non-proliferative.

38. The vaccine of claim 37, wherein said cell line is a human cell line.

39. A composition of matter useful in treating a cancerous condition comprising a non proliferative cell line having expressed on its surface a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1.

40. The composition of matter of claim 39, wherein said cell line is a human cell line.

41. A composition of matter useful in treating a cancerous condition, comprising (i) a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, (ii) an MHC or HLA molecule, and (iii) a pharmaceutically acceptable carrier.

42. Isolated antibody which is specific for the antigen of claim 24.

43. The isolated antibody of claim 42, wherein said antibody is a monoclonal antibody.

44. Method for screening for cancer in a sample, comprising contacting said sample with a nucleic acid molecule which hybridizes to all or part of SEQ ID NO: 1, and determining hybridization as an indication of cancer cells in said sample.

45. A method for screening for cancer in a sample, comprising contacting said sample with the isolated antibody of claim 42, and determining binding of said antibody to a target as an indicator of cancer.

46. Method for diagnosing a cancerous condition in a subject, comprising contacting an immune reactive cell containing sample of said subject to a cell line transfected with the isolated nucleic acid molecule of claim 1, and determining interaction of said transfected cell line with said immunoreactive cell, said interaction being indicative of said cancer condition.

47. A method for determining regression, progression of onset of a cancerous condition comprising monitoring a sample from a patient with said cancerous condition for a parameter selected from the group consisting of (i) NY-ESO-1 protein, (ii) a peptide derived from NY-ESO-1 protein and (iii) cytolytic T cells specific for said peptide and an MHC molecule with which it non-covalently complexes, wherein amount of said parameter is indicative of progression or regression or onset of said cancerous condition.

48. Method of claim 47, wherein said sample is a body fluid or exudate.

49. Method of claim 47, wherein said sample is a tissue.

50. Method of claim 47, comprising contacting said sample with an antibody which specifically binds with said protein or peptide.

51. Method of claim 50, wherein said antibody is labelled with a radioactive label or an enzyme.

52. Method of claim 50, wherein said antibody is a monoclonal antibody.

53. Method of claim 47, comprising amplifying RNA which codes for said protein.

54. Method of claim 53, wherein said amplifying comprises carrying out polymerase chain reaction.

55. Method of claim 47, comprising contacting said sample with a nucleic acid molecule which specifically hybridizes to a nucleic acid molecule which codes for or expresses said protein.

56. Method of claim 47, comprising assaying said sample for shed protein.

57. Method for diagnosing a cancerous condition comprising assaying a sample taken from a subject for a an immunoreactive cell specific for a peptide derived from NY-ESO-1, complexed to an MHC molecule, presence of said immunoreactive cell being indicative of said cancerous condition.

58. Method for treating a subject afflicted with a cancerous condition, comprising:

(i) removing an immunoreactive cell containing sample from said subject,

(ii) contacting the immunoreactive cell containing sample to a cell line transfected with a gene coding for ESO-1 under conditions favoring proliferation of said immunoreactive cells, and

(iii) introducing said immunoreactive cells to said subject in an amount sufficient to alleviate said cancer.

59. Method of claim 46, wherein said immune reactive cell containing sample comprises blood or serum.

60. A method for treating a subject with a cancerous condition, comprising administering to said subject an amount of a cell transfected with (i) a nucleic acid sequence which codes for NY-ESO-1 and (ii) a nucleic acid sequence which codes for an MHC or HLA molecule which presents a peptide derived from NY-ESO-1, wherein said peptide is presented by cells associated with said cancerous conditions, sufficient to alleviate said cancerous condition.

61. Method of claim 60, further comprising treating said cell to render it non-proliferative.

62. Method for treating a subject afflicted with a cancerous condition comprising administering to said subject an amount of a reagent consisting essentially of non-proliferative cells having expressed on their surface non-covalent complex as MHC molecule and peptides derived from ESO-1.

63. Method for treating a subject afflicted with a cancerous condition comprising administering to said subject an antibody which specifically binds to an ESO-1 derived peptide expressed on a cancerous cell associated with said condition, said antibody being coupled to an

anticancer agent, in an amount sufficient to treat said cancerous condition.

64. Method for treating a subject afflicted with a cancerous condition comprising administering to said subject an antibody which specifically binds to ESO-1, said antibody being coupled to an anticancer agent, in an amount sufficient to treat said cancerous condition.

65. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the immunogenic composition of claim 25 in an amount sufficient to prevent onset of said cancerous condition in said subject.

66. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the immunogenic composition of claim 27 in an amount sufficient to prevent onset of said cancerous condition in said subject.

67. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the immunogenic composition of claim 29 in an amount sufficient to prevent onset of said cancerous condition in said subject.

68. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the immunogenic composition of claim 34 in an amount sufficient to prevent onset of said cancerous condition in said subject.

69. Method for preventing onset of a cancerous condition in a subject comprising administering an amount

of the vaccine of claim 36 in an amount sufficient to prevent onset of said cancerous condition in said subject.

70. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the composition of matter of claim 39 in an amount sufficient to prevent onset of said cancerous condition in said sample.

71. Method for preventing onset of a cancerous condition in a subject comprising administering an amount of the immunogenic composition of claim 35 in an amount sufficient to prevent onset of said cancerous condition in said subject.

72. A method for treating a patient with a cancer comprising administering to said patient a therapeutically effective amount of the isolated cancer associated antigen of claim 24.

73. A method for determining efficacy of a vaccine following administration of said vaccine to a subject, comprising determining antibodies in a sample taken from said subject which bind to said vaccine or to a molecule which comprises said vaccine, wherein a change in level of said antibodies after administration of said vaccine relative to said level prior to administration is indicative of efficacy or lack thereof.

74. The method of claim 58, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

75. The method of claim 60, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

76. The method of claim 62, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

77. The method of claim 63, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

78. The method of claim 64, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

79. The method of claim 65, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

80. The method of claim 66, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

81. The method of claim 67, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

82. The method of claim 68, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

83. The method of claim 69, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

84. The method of claim 70, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

85. The method of claim 71, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

86. The method of claim 72, wherein said subject has at least one of antibodies against the protein encoded by the isolated nucleic acid molecule of claim 1 or cytolytic T cells specific for complexes of HLA molecules and a peptide derived from said subject in a body fluid of said subject.

AMENDED CLAIMS

[received by the International Bureau on 21 January 1998 (21.01.98); original claims 1 and 44 amended; remaining claims unchanged (2 pages)]

1. Isolated nucleic acid molecule which encodes a cancer associated antigen, said isolated nucleic acid molecule having a nucleotide sequence, the complementary sequence of which hybridizes, under stringent conditions, to the nucleic acid molecule consisting of the nucleotide sequence set forth in nucleotides 54-593 of SEQ ID NO: 1.

2. The isolated nucleic acid molecule of claim 1, consisting of nucleotides 54-593 of SEQ ID NO: 1.

3. The isolated nucleic acid molecule of claim 1, consisting of anywhere from nucleotide 1 through nucleotide 747 of SEQ ID NO: 1, with the proviso that said isolated nucleic acid molecule contains at least nucleotides 54-593 of SEQ ID NO: 1.

4. Expression vector comprising the isolated nucleic acid molecule of claim 1, operably linked to a promoter.

5. Expression vector comprising the isolated nucleic acid molecule of claim 3, operably linked to a promoter.

6. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 4.

7. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 5.

8. Isolated cancer associated antigen comprising all or part of the amino acid sequence encoded by nucleotides 54-593 of SEQ ID NO: 1.

44. Method for screening for cancer in a sample, comprising contacting said sample with a nucleic acid molecule which specifically hybridizes to all or part of SEQ ID NO: 1, and determining hybridization to SEQ ID NO: 1 as an indication of cancer cells in said sample.

45. A method for screening for cancer in a sample, comprising contacting said sample with the isolated antibody of claim 42, and determining binding of said antibody to a target as an indicator of cancer.

46. Method for diagnosing a cancerous condition in a subject, comprising contacting an immune reactive cell containing sample of said subject to a cell line transfected with the isolated nucleic acid molecule of claim 1, and determining interaction of said transfected cell line with said immunoreactive cell, said interaction being indicative of said cancer condition.

47. A method for determining regression, progression of onset of a cancerous condition comprising monitoring a sample from a patient with said cancerous condition for a parameter selected from the group consisting of (i) NY-ESO-1 protein, (ii) a peptide derived from NY-ESO-1 protein and (iii) cytolytic T cells specific for said peptide and an MHC molecule with which it non-covalently complexes, wherein amount of said parameter is indicative of progression or regression or onset of said cancerous condition.

48. Method of claim 47, wherein said sample is a body fluid or exudate.

49. Method of claim 47, wherein said sample is a tissue.

FIG. 1

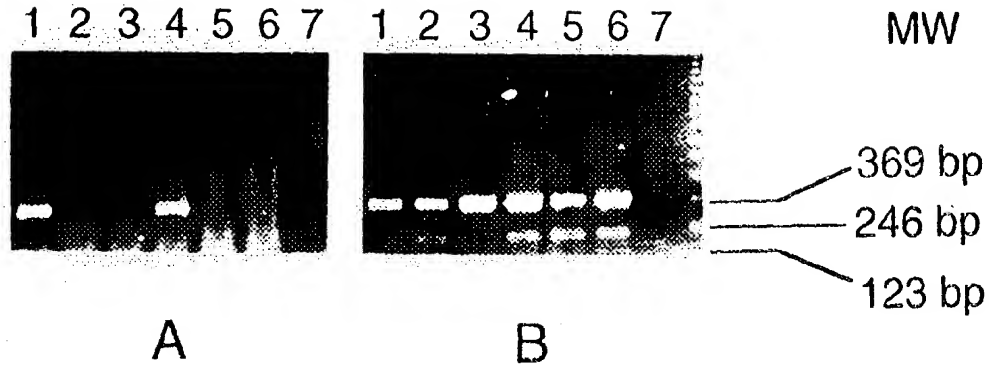

1 2 3 4 5 6 7
1 2 3 4 5 6 7
MW
369 bp
246 bp
123 bp
A
B

FIG. 2

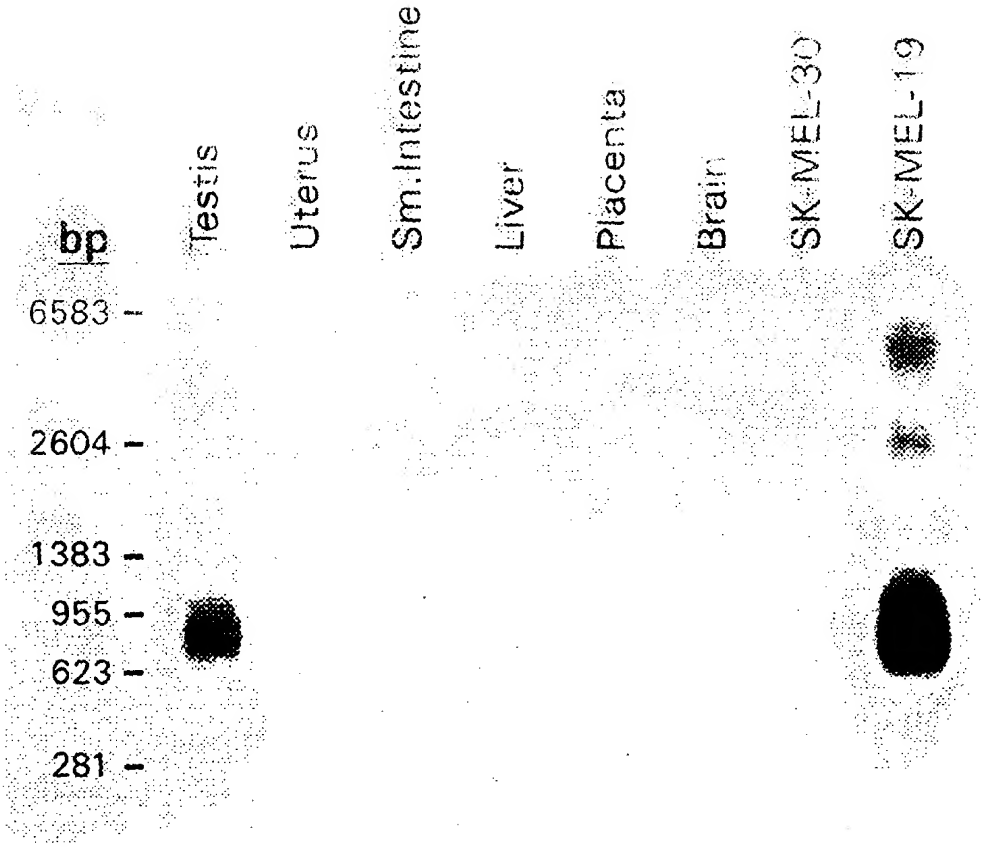

bp
Testis
Uterus
Sm.Intestine
Liver
Placenta
Brain
SK-MEL-30
SK-MEL-19
6583
2604
1383
955
623
281

FIG. 3

```
                ATCCTCGTGGGCCCTGACCTTCTCTCTGAGAGCCGGGCAGAGGCTCCGGAGC
                          Myr  Myr  (P)
                          |    |    |
  M  Q  A  E  G  R  G  T  G  G  S  T  G  D  A  D  G  P  G  G
CATGCAGGCCGAAGGCCGGGGCACAGGGGGTTCGACGGGCGATGCTGATGGCCCAGGAGG

  P  G  I  P  D  G  P  G  G  N  A  G  G  P  G  E  A  G  A  T
CCCTGGCATTCCTGATGGCCCAGGGGGCAATGCTGGCGGCCCAGGAGAGGCGGGTGCCAC

  G  G  R  G  P  R  G  A  G  A  A  R  A  S  G  P  G  G  G  A
GGGCGGCAGAGGTCCCCGGGGCGCAGGGGCAGCAAGGGCCTCGGGGCCGGGAGGAGGCGC

  P  R  G  P  H  G  G  A  A  S  G  L  N  G  C  C  R  C  G  A
CCCGCGGGGTCCGCATGGCGGCGCGGCTTCAGGGCTGAATGGATGCTGCAGATGCGGGGC

                                                      (P)
                                                      |
  R  G  P  E  S  R  L  L  E  F  Y  L  A  M  P  F  A  T  P  M
CAGGGGGCCGGAGAGCCGCCTGCTTGAGTTCTACCTCGCCATGCCTTTCGCGACACCCAT

  E  A  E  L  A  R  R  S  L  A  Q  D  A  P  P  L  P  V  P  G
GGAAGCAGAGCTGGCCCGCAGGAGCCTGGCCCAGGATGCCCCACCGCTTCCCGTGCCAGG

                                                (P)          (P)
                                                |            |
  V  L  L  K  E  F  T  V  S  G  N  I  L  T  I  R  L  T  A  A
GGTGCTTCTGAAGGAGTTCACTGTGTCCGGCAACATACTGACTATCCGACTGACTGCTGC

  D  H  R  Q  L  Q  L  S  I  S  S  C  L  Q  Q  L  S  L  L  M
AGACCACCGCCAACTGCAGCTCTCCATCAGCTCCTGTCTCCAGCAGCTTTCCCTGTTGAT

  W  I  T  Q  C  F  L  P  V  F  L  A  Q  P  P  S  G  Q  R  R
GTGGATCACGCAGTGCTTTCTGCCCGTGTTTTTGGCTCAGCCTCCCTCAGGGCAGAGGCG

*
CTAAGCCCAGCCTGGCGCCCCTTCCTAGGTCATGCCTCCTCCCCTAGGGAATGGTCCCAG
CACGAGTGGCCAGTTCATTGTGGGGGCCTGATTGTTTGTCGCTGGAGGAGGACGGCTTAC
ATGTTTGTTTCTGTAGAAAATAAAACTGAGCTACGAAAAA
```

FIG. 4

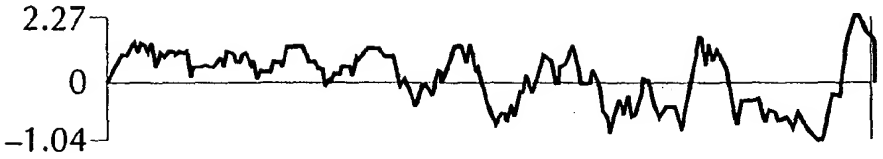


FIG. 5

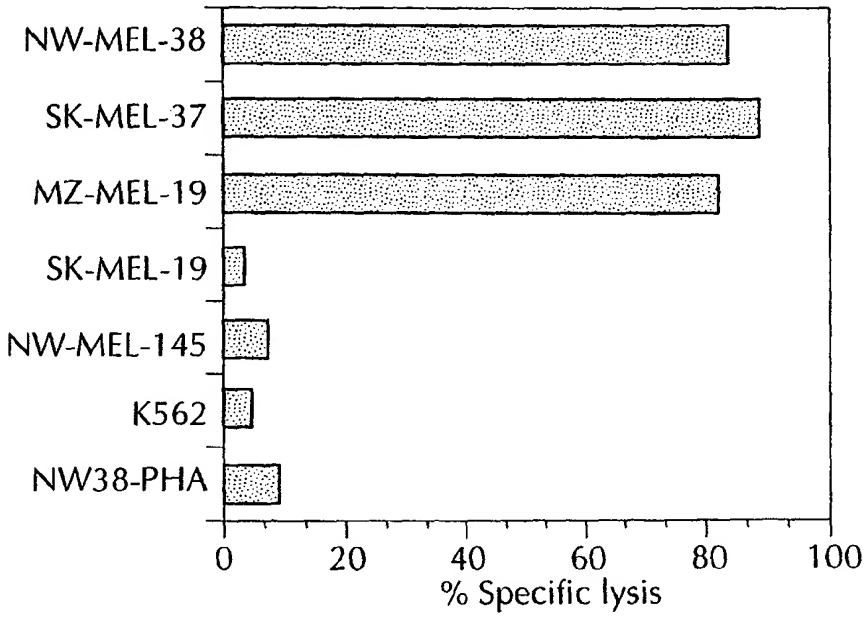
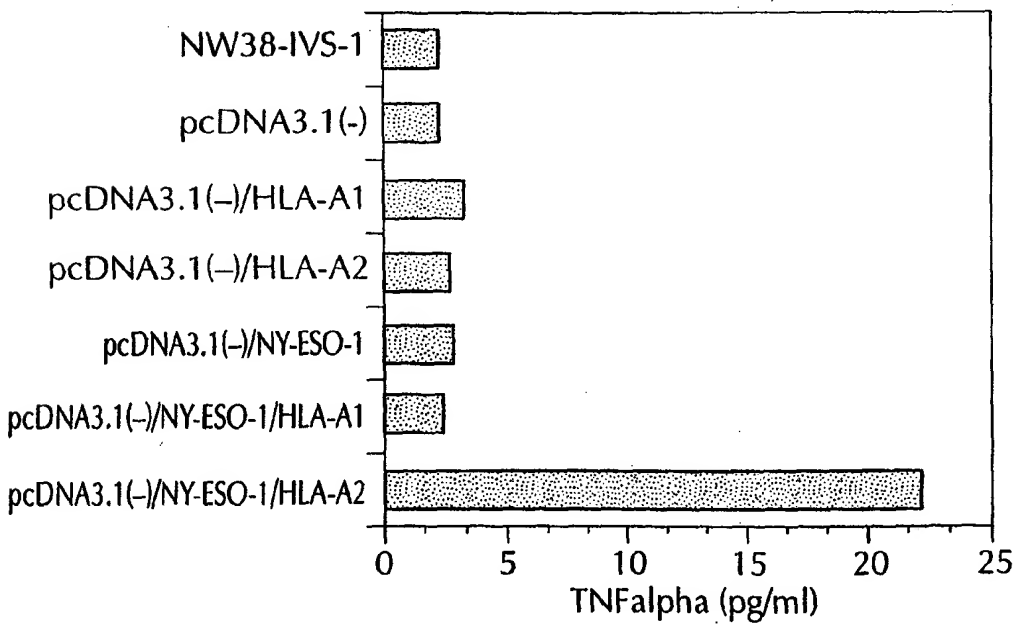


FIG. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16335

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21; 435/6, 7.1, 91.2, 240.2, 320.1; 514/2, 44; 530/350, 387.1, 388.1; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KIYOKAWA et al. Overexpression of ERK, an EPH Family Receptor Protein Tyrosine Kinase, in Various Human Tumors. Cancer Research. 15 July 1994, Vol. 54, No. 14, pages 3645-3650, especially abstract, Figure 1 and pages 3649.	1,4,6,12
A, P	CHEN et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc. Natl. Acad. Sci. USA. March 1997, Vol. 94, No. 5, pages 1914-1918, see entire document.	1-86

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 OCTOBER 1997	Date of mailing of the international search report 04 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DEBRA SHOEMAKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/16335

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FAZIOLI et al. The ezrin-like family of tyrosine kinase substrates: receptor-specific pattern of tyrosine phosphorylation and relationship to malignant transformation. Oncogene. May 1993, Vol. 8, No. 5, pages 1335-1345, especially abstract.	1-33
A	US 5,212,085 A (WANDS et al) 18 May 1993, especially abstract and col. 8-10 and 19-20.	1-86